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Complete List of Authors:	Su, Yuping; Fujian Normal University, Zhang, Dayi; Lancaster University, Lancaster Environment Centre
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Recruitment of cyanobacteria from the sediments in the eutrophic Shanzi Reservoir

Yuping Su[1,2], Xuejing You[1], Hui Lin[1], Huiru Zhuang[1], Yuan Weng[1], Dayi Zhang[1,2,*]

1. Environmental Science and Engineering College, Fujian Normal University, Fuzhou, China PR, 350007

2. Lancaster Environment Centre, Lancaster University, Lancaster, UK, LA1 4YW

Correspondence to: Dayi Zhang

Lancaster Environment Centre, Lancaster University

Lancaster, UK, LA1 4YW

E-mail: d.zhang@lancaster.ac.uk

Abstract

This study investigated the impacts of four environmental factors on the recruitment of cyanobacteria from the bottom sediments in the eutrophic Shanzi Reservoir. Temperature and light were identified as the key determinants for the recruitment of *Microcystis* and *Oscillatoria*. Cyanobacteria became dominant at higher temperature (20ºC) and light intensity (2,000 lx), and *Microcystis* and *Oscillatoria* were the major species. Detailed recruitment simulation undertaken with respective gradient of temperature and light suggested that both *Microcystis* and *Oscillatoria* are temperature sensitive, and their critical temperature point was 10ºC. However, distinct light impacts were observed only on *Microcystis*. The recruitment of *Oscillatoria* was light independent, whereas *Microcystis* had positive relationship with light intensity. Physical disturbance promoted *Microcystis* recruitment and also affected the structure of recruited cyanobacterial community the water-sediment interaction, based on quantitative polymerase chain reaction (qPCR) and phylogenetic analysis.

Keywords

eutrophication, *Microcystis*, *Oscillatoria*, sediment, recruitment, quantitative PCR

1. Introduction

China has recently suffered from not only shortage of water resources to support the growing number of cities, but also severe water contamination [1]. With the rapid economic development and associated human activities, the contaminants from industry, agriculture and domestic wastes have consequently resulted in the deterioration of water quality in reservoirs. It is reported that one third of Chinese important reservoirs are or are becoming eutrophic [1]. As the consequence of water eutrophication, cyanobacteria blooms, particularly the toxigenic cyanobacteria like *Microcystis*, directly threaten the drinking water safety [2].

The growth and formation of cyanobacterial blooms were suggested to have four stages, including featuring dormancy, recovery, increasing biomass and floating upward to form water blooms [3]. Cyanobacteria recruitment has been regarded as a process where benthic overwintering cyanobacteria migrate to the pelagic phase and renew growth under suitable conditions [4]. Increasing more research has attempted to reveal the key factors affecting the recruitment of cyanobacteria. Those environmental parameters, besides nutrients as nitrogen and phosphate, showed significant impacts on the recruitment and population fluctuation of cyanobacteria, as well as the structure and functions of microbial community [5]. More interestingly, cyanobacteria might be dominant but inert at the water-sediment interface when environmental conditions are harsh for recruitment like low temperature or light limitation, until rapidly growing under suitable conditions, a so-called *rejuvenation* phase [6]. It is therefore important to uncover the factors affecting the accumulation and distribution of cyanobacteria at the water-sediment interface, and the determinants contributing to the recruitment process.

Since the exposure to microcystin was highly associated with primary liver cancer in some areas of China [7], the microcystin producing cyanobacterial genus, *Microcystis*, has drawn increasingly more attention [8]. Some environmental factors affected *Microcystis* recruitment in natural environment, including temperature, light, nutrients, dissolved oxygen, physical resuspension and bioturbance [9, 10, 11, 12]. *Microcystis*

was reported to become active in sediments when the temperature of deep lakes reached 7 to 8 ºC [13], and their growth rate increased significantly at 15ºC. On the contrary, though the growth rate of *Microcystis* was sensitive to light intensity, its recruitment process is not light-sensitive [12, 14]. The low level of photosynthesizing efficiency and electron transfer rate can only restrict their growth rate. However, the key restricted factors vary in lakes from different regions, and it is therefore important to uncover the key environmental determinants in specific lake conditions for practical water quality management.

This research introduced orthogonal recruitment experiments to address the determinants affecting the cyanobacteria recruitment in the sediments of Shanzi Reservoir (China), which is a classic subtropical small reservoir. Four environmental factors, temperature, light intensity, physical disturbance and nutrients, were experimentally manipulated to evaluate the contribution of sediment to the recruitment process of planktonic populations in this eutrophic reservoir [12, 14, 15, 16]. Detailed study of quantitative PCR and microbial community at water-sediment interface also provided the *in situ* dynamic information on *Microcystis* recruitment from sediment into the water body in Shanzi Reservoir.

2. Experimental details

2.1 Sampling site

Shanzi Reservoir was built in 1992 and is one of the important drinking water resources of Fuzhou district, Fujian Province, China (Figure 1). It is located within the catchment of Aojiang River, which is the sixth largest river in Fujian Province, 137 km length and with a 2,655 km^2 watershed. Shanzi Reservoir has regulation storage of 1.06×10^8 m^3 with a surface area of 6.639 km^2 and average depth of 25 m. The upriver catchment area of Shanzi Reservoir is 1,646 km^2 with an annual average depth runoff of 1.857×10^9 m^3 respectively [17]. With integrated usages of irrigation, electricity generation, water supply and flood control, Shanzi Reservoir has experienced severe eutrophication. The algal blooms occur every year from May to October, and the

conditions and extent of its outbreak in late spring determine the level and scale of algal bloom in the following months [17]. The dominant species are cyanobacteria, especially *Microcystis*, with strong impacts on water quality [2].

2.2 Samples collection and analysis

During the algal bloom period (May to October), numerous recruited cyanobacteria could be found in both sediment and water samples and their existence affected the evaluation on environmental factors affecting cyanobacterial recruitment. Thus, the sediment and water samples were taken in Shanzi Reservoir (26°20'22"N and 119°19'48"E, 10 m depth) in November 2010. A total 2.0 L of water-sediment interface water was collected within 20 cm distance above the sediment. Subsequently, the sixteen sediment cores were collected from 0 cm to 10 cm in the sediment by the Kajak sediment corer (6 cm in diameter) for further chemical and biological analysis (UWITECH, Mondsee, Austria). Both water and soil samples were stored in the plexiglass tubes (diameter 6 cm and height 25 cm) at 4°C for further recruitment experiment. The 1.0 L of water-sediment interface water was filtered by 0.45 μm filter, (Millipore, USA) to removal residual cyanobacteria and suspended solids. Filtered water pH value (6.5-7.0) was measured by pHS-3C series pH Meter (Shanghai REX Instrument Factory, China). The total nitrogen (TN) in water sample was directly determined by $K_2S_2O_8$ spectrophotometric method, and the total phosphate (TP) was measured by phosphatic molybdenum spectrophotometry [18]. For sediment samples, TN and TP were determined by vario EL III Element Analyzer (Elementar, Germany) and molybdenum blue/ascorbic acid method [19].

2.3 Recruitment experiment

The orthogonal experiments addressed the impacts of light, temperature, nutrients and disturbance on cyanobacterial recruitment, the different treatments of which were shown in Table 1. Briefly, 15 g of sediment samples was transferred into a cylindrical glass column for recruitment simulation. In accordance with the annual spring temperature variation of Shanzi Reservoir (10°C to 20°C), the two temperature levels in recruitment experiment were 10°C and 20°C, representing early and late spring

respectively. The two light intensity conditions included the high light treatment of 2,000 lx and low light treatment 50 lx. The dark condition was achieved by covering the light source with kraft paper, and a 12 h:12 h light-dark-cycle was used to simulate natural conditions. Two recruitment medium (25 mL), filtered water-sediment interface water or BG11 medium, were furnished with the sediment to investigate the impacts of nutrients. The BG11 medium contained 17.6 mM $NaNO_3$, 0.22 mM K_2HPO_4, 0.3 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.03 mM citric acid, 0.02 mM ferric ammonium citrate and 0.002 mM Na_2EDTA, 0.18 mM Na_2CO_3 and 1 mL L^{-1} BG11 trace metal solution [20]. The pH value of BG11 medium was adjusted at 6.5-7.0 by adding 0.1 M NaOH or HCl. Physical disturbance was simulated with a shaker, which provided 60 rpm vertical shaking for 30 minutes at 09:00 h, 15:00 h and 21:00 h each day, simulating the sluice activities in the field. Each treatment was set up with three replicates and cultivated in PGXX-350B (Fuma, China) and PGX-160C (Fuma, China) intelligent incubator. From preliminary test, significant cyanobacterial recruitment was observed within 4 days from sediments [2], and the recruitment experiment in this study therefore was carried out for 6 days. The recruitment experiments were carried out in two separate time periods as biological replicates.

To address the impacts of temperature and light on the recruited algal community structure, further experiment was set up under six temperatures (6, 8, 10, 12, 14 and 16ºC) and two light conditions (2,000 and 50 lx). The 200 mL filtered water-sediment interface water was transferred into PGX-160C intelligent biochemical incubator to cover the six sediment cores. After recruitment for 6 days, the 200 mL of the supernatant was subsequently collected for further algae counting and molecular biological analysis.

2.4 Cyanobacterial community analysis by algae counting

The algal species in water solution were identified with the binocular biological microscope (Motic, BM-1000, Guangzhou) [21]. Briefly, 300 μL Lugols iodine solution was added into 20 mL water sample, concentrated by centrifugation to the final volume of 100 μL. The 0.1 mL counting chamber (20 mm×20 mm) was utilized

for microalgae identification and counting, and three replicates were applied. All the samples were measured at 4°C under dark condition, and the microalgae counts were converted to the cells per unit (cells/mL) by the following equation.

$$N = (\frac{A}{A_0} \times \frac{1}{V}) \times n$$

Here, N refers to microalgae counts per millilitre water sample (cells mL^{-1}). A represents the area of counting chamber (mm^2), and A_0 was the counting area (mm^2). V is the volume of counting chamber (0.1 mL), and n refers to the number of microalgae within the counting area (cells). For multicellular filamentous *Oscillatoria*, the number was counted by individual cells under microscope.

To determine the microalgae counts in sediment samples, the 1.0 g of fresh sliced samples from the water-sediment interface were mixed well with 100 mL deionized water in clean breakers. The upper 30 mL suspension was taken and added with 450 µL Lugols iodine solution and 1.27 mL formaldehyde solution (final concentration of 4%, V/V). The algal counting and calculation followed the same instruction as described in water sample analysis.

2.5 PCR amplification for specific gene fragments

Genomic DNA of sediment samples was extracted with CTAB methods as described previously [22]. All the primers applied in this research were designed in accordance with previous research and synthesized by VWR International LLC, as listed in Table 2. All the PCR programs were undertaken in C1000 Thermal Cycler (BioRad, USA), and the reaction system (50 µL) contains 2.5 µL of each primer, 0.5 µL DreamTaq DNA polymerase (Thermo Scientific, USA), 5 µL DreamTaq green buffer (Thermo Scientific, USA), 1 µL dNTPs (5 mM), 1 µL DNA template and 41 µL molecular water (Sigma Aldrich, USA). Three groups of primers were designed to address the quantification of microcystin synthetase genes (*mcyA* and *mcyB*), bacterial 16S rRNA and cyanobacterial 16S rRNA. For *mcyA* microcystin synthetase genes (mcyA_f and mcyA_r), the PCR program consisted of an initial stage of 5 min at 94 °C, followed by 40 cycles of 10 s at 94°C, 20 s at 50°C and 60 s at 72°C, and the final extension at

72°C for 5 min [23]. For the *Microcystis*-specific 16S rRNA and *mcyB* microcystin synthetase genes, Nested PCR was applied with respective primers [24]. For the first generation PCR, including 16S_f1/16S_r1 for *Microcystis*-specific 16S rRNA and MCY_f1/MCY_r1 for *mcyB* microcystin synthetase genes, the initial denaturation is 30 s at 94°C, followed by 45 cycles of 30 s at 94°C, 45 s at 57°C and 60 s at 72°C, with a final extension of 10 min at 72°C [25]. For the second generation for *Microcystis*-specific 16S rRNA (16S_f2/16S_r1) and *mcyB* microcystin synthetase genes (MCY_f1/MCY_r2), the initial denaturation was 30 s at 94°C, followed by 40 cycles of 30 s at 94°C, 45 s at 57°C and 60 s at 72°C, with a final extension of 10 min at 72 °C [25]. For the cyanobacteria-specific 16S rRNA genes (209f and 409r), the initial denaturation was 94°C for 4 min, followed by 40 cycles of 94°C for 20 s, 50°C for 30 s and 72°C for 120 s, with the final extension at 72°C for 5 min [26]. The PCR program for total bacteria 16S rRNA genes (63f/1387r and 519f/907r) consisted of an initial denaturation of 4 min at 95°C, followed by 40 cycles of 30 s at 95°C, 60 s at 58°C and 120 s at 72°C, and the final extension at 72°C for 5 min [27, 28]. The PCR products of *mcyA* microcystin synthetase fragments were ligated into the pGEM-T vector (Promega, USA), transferred into *E. coli* JM109 competent cells by heat shock, and then selected on LB agar with 300 mg/L ampicillin as the antibiotic pressure. The plasmids with specific *mcyA* fragment of the positive clones were extracted and sent for sequence.

2.6 Quantitative PCR

Quantitative PCR was applied to quantify the copies of *Microcystis*-specific 16S rRNA and *mcyB* microcystin synthetase genes in sediment samples with CFX96 Real-Time PCR Detection System (BioRad, USA). The reaction system (10 μL) consisted of 5 μL iTAQ SYBR-green supermix (BioRad, USA), 1.0 μL of each primer, 1.0 μL DNA template and 2.0 μL molecular water. The program of each reaction was identical to that described above for each pair of primers, with additional melting curve detection from 65 °C to 95 °C at 0.5 °C intervals. All the amplified DNA fragments were purified with the Gel Extraction Kit (QIAGEN, USA). The DNA

fragments were subsequently cloned into the pGEM®-T vector (Promega, USA) following the manufacturer's instructions. Plasmid DNA was then extracted and purified with Minipreps Kit (Promega, USA) and the inserts were confirmed by PCR with the respective program. The DNA concentration was determined with Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Invitrogen, USA) with 480 nm excitation and 520 nm detection wavelength, by Synergy 2 plate reader (BioTek, USA). The plasmid copy number of each DNA insert was determined by the amount and molecular weight of the targeting double-stranded plasmid [22]. All the plasmids were diluted in the series of 3×10^8, 3×10^7, 3×10^6, 3×10^5, 3×10^4, 3×10^3, 3×10^2, 3×10^1 and 3×10^0 copies for each reaction, quantified together with the targeting environmental samples in the same qPCR program to obtain the linear standard curve, as illustrated in Table 3 and Figure S1.

2.7 Data analysis

SPSS package (version 11.0) was used for statistical analysis by ANOVA analysis of all the variance. All the data were the values are the means of data from three independent replicates and the *p*-value <0.05 was considered as statistical significance. The phylogenetic tree of *mcyA* genes (*Shanzi S1*, *Shanzi S2* and *Shanzi S3*) from Shanzi Reservoir was calculated and drawn in MEGA 4.0 (http://www.megasoftware.net/mega4/mega.html), and compared with 20 known *mcyA* genes of *Microcystis sp.* from NCBI database (http://www.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1 Impacts of temperature and light on cyanobacteria recruitment

The results of recruitment experiments (Table 1) showed that *Microcystis* and *Oscillatoria* were the two primary recruited cyanobacteria from the sediments. Of all the factors tested in this study, the determinants regulating the recruitment of *Microcystis* were identified as temperature, light and physical disturbance from ANOVA analysis (Table S1). Under low temperature (10°C) and light (50 lx) conditions, the population of *Microcystis* was only 252±15 cells mL^{-1}, whereas they

increased 40 times (9,934±397 cells mL^{-1}) at 20°C and 2,000 lx after 6 days. More precisely from the analysis of variance (Table 1), *Microcystis* recruitment was stimulated by the temperature (averagely 1,321 cells mL^{-1} at 10°C and 5,941 cells mL^{-1} at 20°C) and light (averagely 1,950 cells mL^{-1} with 50 lx and 5,312 cells mL^{-1} with 2,000 lx) respectively. As for the impacts of physical disturbance, *Microcystis* count was higher in disturbance treatments (3,271 cells mL^{-1}) than in no disturbance treatments (630 cells mL^{-1}). The possible reason was the enhancement of nutrients distribution and cell-mineral interaction to accelerate recruitment process [29, 30]. The similar *Microcystis* count (3,742 cells mL^{-1} in raw water and 3,520 cells mL^{-1} in BG11 medium) showed limited effect of nutrients in this study. The optimal conditions for *Microcystis* recruitment were therefore identified as 20°C and 2,000 lx with physical disturbance. The recruitment of *Oscillatoria* was significantly slower than *Microcystis* (Table S2), and the average populations were 1325±68 cells mL^{-1} under 20°C and 2,000 lx condition (Table 1). The analysis of variance only illustrated one key factor, temperature, affecting *Oscillatoria* recruitment (averagely 334 cells mL^{-1} at 10°C and 1,457 cells mL^{-1} at 20°C).

Cultivated in different temperature conditions, more evidence was found on the impacts of temperature and light on the recruitment rate of *Microcystis* and *Oscillatoria* (Figure 2). A significant lag phase was observed for both strains at 8°C. The positive relationship was found between the temperature and *Microcystis*/*Oscillatoria* from 10°C to 16°C, and the correlation was similar to previous investigation on the impacts of temperature on cyanobacteria growth from 20°C to 35°C [31]. The results indicated that temperature is a sensitive factor for *Microcystis*/*Oscillatoria* in Shanzi Reservoir under late spring conditions. There was no significant difference for *Oscillatoria* recruitment under different light conditions (Figure 2b).

The results were similar to previous research that *Microcystis* was sensible to the light for recruitment [32] and *Oscillatoria* were less light sensitive [33]. Given the interspecific competitive recruitment and the annual spring temperature in Shanzi Reservoir (10°C to 20°C), it explained the dominancy of *Microcystis*, rather than

Oscillatoria, in the blooms. Under this optimal condition [30], *Microcystis* became the dominant cyanobacterial species during the eutrophication in South China, previously reported to constitute 95% of the total cyanobacterial population in Taihu Lake [34]. It also explained that *Microcystis* was the dominant species in the sediments in winter while in the water suspension in summer [35].

3.2 Cyanobacterial community structure at water-sediment interface

From the microbial community analysis of original water and sediment samples, cyanobacteria and diatoms (62.5% and 37.5% respectively) were dominant at the water-sediment interface, whereas *Chlorella* were hardly found in water phase (less than 1%) but mainly in the water body (5.3%, Figure 3a). The cyanobacteria in the sediment microbial community decreased with the increasing light intensity as 2.7% and 5.4% for 2,000 lx and 50 lx treatments respectively (Figure 3b and 3c). No significant change was observed for diatom (89.8%, 91.0% and 92.0% in original, 2,000 lx and 50 lx treatments, p-value>0.05) and *Chlorella* (5.3%, 3.6% and 5.3% in original, 2,000 lx and 50 lx treatments, p-value>0.05) profiles in sediment at different levels. At the water-sediment interface, high light treatment (2,000 lx) significantly improved cyanobacterial recruitment (from 62.5% to 71.4%), whereas diatoms became dominant in low light treatment (50 lx, from 37.5% to 64.0%). No *Chlorella* recruitment was observed (less than 1% of the total population). From previous evidence, cyanobacteria had the chlorophyll and phycobiliprotein to utilize the light with wavelength from 500 nm to 600 nm [36], which is not valid for other algal species. Thus, the high efficiency of cyanobacterial photosynthesis activity contributed to its dominancy under high light conditions [16, 37, 38]. The intra-cellular carbohydrate of *Oscillatoria* possibly provided enough energy to recruit, as stated previously in Taihu Lake [36].

From the annual cycle of *Microcystis sp.* at the water-sediment interface, the low temperature and light intensity in winter therefore was not suitable condition for cyanobacteria recruitment. It consequently resulted in different community structure at the water-sediment interface and main water body. During the winter when the

temperature in Shanzi Reservoir was estimated to be 10°C, the *Microcystis* recruitment was inhibited in the water body [39] and *Microcystis* mainly existed in the sediment [35]. After carbohydrate accumulation in winter, *Microcystis* started the effective photosynthesis in late spring when the temperature is above 10°C, further leading to their dominancy in water phase [40, 41, 42]. Compared with previous field simulation of sediment *Microcystis* recruitment in Dianchi Lake [43] and Hirosawa-no-ike Pond [44], the strong positive relationship between monthly temperature change and the concentration of *Microcystis* in water sample has suggested that temperature is the key environmental factor affecting *Microcystis* recruitment at the water-sediment interface.

Sediment disturbance had significant impacts on cyanobacteria recruitment in this study. The frequent water-sediment interface disturbance and mixture in shallow lake helped in forming complex community structure, promoting cell-cell or cell-mineral interaction to accelerate recruitment process [29, 30]. Different from the key factors in other lakes with fewer depth [45], this study indicated the importance of microbial community in cyanobacterial recruitment and bloom formation in deep lake, instead of the weak historical and physical disturbance. The remarkable effects of physical disturbance on *Microcystis* and *Oscillatoria* in this work explained the better recruitment of cyanobacteria under disturbance, fitting well with physiological research on *Microcystis* recruitment [46, 47]. The aerobic environment created by physical disturbance benefited their resuscitation from the sediment into water body [48]. It was reported that the single vertical convection type of water temperature in Shanzi reservoir could form a stable thermal stratification from March to November [49]. The stratification disappearance from December to next February promoted vertical mixture, allowing the release of recruitment cyanobacteria from the sediment to the surface water, contributing to the planktonic populations and community succession.

No significant impact of nutrient was observed here due to minimal difference of TN/TP ratio between the BG11 medium and the bottom raw water. Bottom raw reservoir water contained 1.369 mg L^{-1} total nitrogen and 0.056 mg L^{-1} total

phosphate, with the TN/TP ratio as 24.4, whereas BG11 selective culture medium contained 247 mg L^{-1} total nitrogen and 7.09 mg/L total phosphate, with the TN/TP ratio as 34.8. The average nitrogen concentration in the sediment of Shanzi Reservoir was around 3,000 mg kg^{-1} with the major type of organic nitrogen, whereas the main phosphorus concentration consisted of active Fe/Al-P and organic phosphorus with the load of 600 to 1,000 mg kg^{-1}. Obviously, the sediment of Shanzi Reservoir stored enough nutrients, especially the essential nitrogen sources [50], to support the recruitment of cyanobacteria, as the BG11 medium.

3.3 Molecular quantification of cyanobacteria and toxic Microcystis

Besides environmental physical factors, the cyanobacterial community structure is also affected by the existence of harmful *Microcystis*. As illustrated in Table 3, bacteria was the dominant species in the sediment with the 16S copy number at $(4.4\pm0.03)\times10^9$ g^{-1} dry sediment. The amount of cyanobacteria 16S rRNA was of high level at $(3.6\pm0.31)\times10^6$ copies g^{-1} dry sediment, which is equivalent to the cyanobacteria bloom occurred in Singapore [24, 51]. With averagely 2 to 4 copies of 16S rRNA genes in cyanobacterial cells [52], the estimated cyanobacteria in sediment were 0.9×10^6 to 1.8×10^6 cells g^{-1} dry sediment. *Microcystis* had only one copy of *mcyA* and *mcyB* microcystin synthetase gene [53], indicating that the toxic cyanobacteria was around 1.2×10^5 to 2.1×10^5 cells g^{-1} dry sediment. The microcystin producing cyanobacteria therefore held 6.9% to 23.4% of total cyanobacteria in sediment. Compared with the numbers (6.0×10^5 cells g^{-1} dry sediment) calculated with two 16S rRNA copies in one *Microcystis* cell, 20.0% to 35.0% of total *Microcystis sp.* could produce toxic microcystin. The lower *mcyA* copies in toxic cyanobacteria might be explained by the *mcyHA* deletion in the proportion of inactive *mcy* genotypes [54]. The results also indicated that the spatial and temporal diversity of microcystin producing cyanobacteria are highly associated with the environmental parameters in sediment samples [7], and the cyanobacteria with active *mcyA* and *mcyB* genotypes were the dominant species in planktonic population, which has been proved in many cases [22, 26, 54, 55].

Compared to quantitative analysis by flow cytometer [56], quantitative PCR identified and quantified *Microcystis sp.* at molecular biological level. Instead of the 16S or 23S rRNA analysis, the phylogenetic relationships of *mcyA* genes (Figure 4) indicated three dominant toxic *Microcystis sp.* after recruitment from Shanzi Reservoir sediments. *Shanzi S1* had high similarity (99%) with *M. aeruginosa* FCY-28 and FCY-26 [57], suggesting its strong capability to produce microcystin toxins and compete with other nontoxic cyanobacteria in seasonal cyanobacteria bloom [58]. *Shanzi S2* was similar (98%) to *M. botrys* N-C 161/1 and *M. viridis* N-C 169/7, whereas *Shanzi S3* had the high similarity (95%) with *M. aeruginosa* SPC777 and *M. aeruginosa* PCC 7820. Previous morphological data support the results that *Microcystis* was the dominant cyanobacteria in the eutrophic Shanzi Reservoir [2], due to their inhibitory impacts on other species caused by microcystin toxins [59, 60]. Besides, the diatom-cyanobacterial symbioses was also observed during the recruitment process (data not shown), indicating the contribution of diatom to the nitrogen fixation process of cyanobacteria could also promote *Microcystis sp.* recruitment [61]. The competition and symbiosis were recognized as another key factor affecting the recruitment of cyanobacteria [62].

4. Conclusions

This research has revealed that temperature, light and physical disturbance are the most important determinants regulating the cyanobacterial recruitment in early spring algal bloom in Shanzi Reservoir, whereas no significant impacts of nutrients were found due to its excess amount in the sediments. High temperature and light intensity stimulated cyanobacterial recruitment from the sediment. *Oscillatoria* recruitment was only sensitive to temperature, and the critical recruitment temperature was 10 ºC for both *Microcystis* and *Oscillatoria*. *Microcystis* was also dominant during recruitment process, due to its utilization of specific light wavelength. While at the natural sediment and water interface, physical disturbance and nutrients were becoming more important for cyanobacterial recruitment. The water stratification in Shanzi Reservoir was interrupted in late spring, when vertical water mixture and

water-sediment interface disruption significantly affected the composition and succession of the phytoplankton community. Both competition and symbiotic within the sediment community determined the structure of microbial and phytoplankton community, and the phylogenetic microcystin synthetase genes (*mcyA*) of *Microcystis sp.* were suggested as the key indicator to explain the respective contribution of various environmental factors during the recruitment process.

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Table

Table 1. Determinants analysis of *Microcystis* and *Oscillatoria* recruitment from the sediment of Shanzi Reservoir.

NO.	Temperature (°C)	Light (lx)	Disturbance	Nutrient	*Microcystis* (cells mL^{-1})	*Oscillatoria* (cells mL^{-1})
1	10	50	-	Raw	252±15	252±10
2	10	50	-	BG11	1,007±50	168±8
3	10	2,000	+	Raw	2,768±138	252±12
4	10	2,000	+	BG11	1,258±58	662±35
5	20	50	+	Raw	2,013±101	1,258±50
6	20	50	+	BG11	4,529±182	1,258±65
7	20	2,000	-	Raw	9,934±397	1,325±68
8	20	2,000	-	BG11	7,285±219	1,987±97
K_1 (*M.*)*	1,321	1,950	630	3,742		
K_2 (*M.*)*	5,941	5,312	3,271	3,520		
K_1 (*O.*)*	334	734	933	772		
K_2 (*O.*)*	1,457	1,056	858	1,019		

* K_1 represents the experimental condition of 10°C, 50 lx, no disturbance and raw water; K_2 represents the experimental condition of 20°C, 2,000 lx, disturbance and BG11 medium.

Table 2. Primers for total bacteria, cyanobacteria and *Microcystis*.

Primer	Sequence (5'-3')	Reference
mcyA_f	ATCCAGCAGTTGAGCAAGC	[23]
mcyA_r	TGCAGATAACTCCGCAGTTG	[23]
MCY_f1	TGGGAAGATGTTCTTCAGGTATCCAA	[25]
MCY_r1	AGAGTGGAAACAATATGATAAGCTA	[25]
MCY_r2	GAGATCCATCTGTTGCAAGACATAG	[25]
16S_f1	CGCAATGGGCGAAAGCCTGACGGAGC	[25]
16S_f2	CCGCGTGAGGGAGGAAGGTCTTTG	[25]
16S_r1	GCGTGCGTACTCCCCAGGCGGGATAC	[25]
209f	ATGTGCCGCGAGGTGAAACCTAAT	[26]
409r	TTACAATCCAAAGACCTTCCTCCC	[26]
63f	CAGGCCTAACACATGCAAGTC	[27]
1387r	GGGCGGWGTGTACAAGGC	[27]
519f	CAGCMGCCGCGGTAANWC	[28]
907r	CCGTCAATTCMTTTRAGTT	[28]

The primer pair 16S_f2/16S_r1 for *Microcystis*-specific 16S rRNA amplification; mcyA_f/mcyA_r for *mcyA* microcystin synthetase gene amplification; MCY_f1/MCY_r2 for *mcyB* microcystin synthetase gene amplification; 209f/409r for cyanobacteria-specific 16S rRNA amplification; 63f/1387r for total bacteria 16S rRNA amplification. For quantitative PCR program, the primers for *Microcystis*-specific 16S rRNA, *mcyA* microcystin synthetase gene, *mcyB* microcystin synthetase gene, cyanobacteria-specific 16S rRNA and total bacteria 16S rRNA are 16S_f1/16S_r1, mcyA_f/mcyA_r, MCY_f1/MCY_r1, 209f/409r and 519f/907r, respectively.

Table 3. Copies of 16S and functional genes of total bacteria, cyanobacteria and *Microcystis* in the sediments of Shanzi Reservoir.

	Copies/(g dry sediment)	Efficiency	Slope	r^2
Total bacteria 16S	$(4.4\pm0.03)\times10^9$	106.00%	-3.186	0.9979
Cyanobacteria 16S	$(3.6\pm0.31)\times10^6$	98.15%	-3.367	0.9935
***Microcystis* 16S**	$(1.2\pm0.03)\times10^6$	98.68%	-3.354	0.9908
mcyA	$(2.1\pm0.14)\times10^5$	102.26%	-3.269	0.9917
mcyB	$(1.2\pm0.12)\times10^5$	101.78%	-3.280	0.9979

Note: The efficiency (between 90% and 110%) and slope (between -3.58 and -3.10) were satisfied for the quantification of targeting 16S and functional genes in environmental samples.

Figure

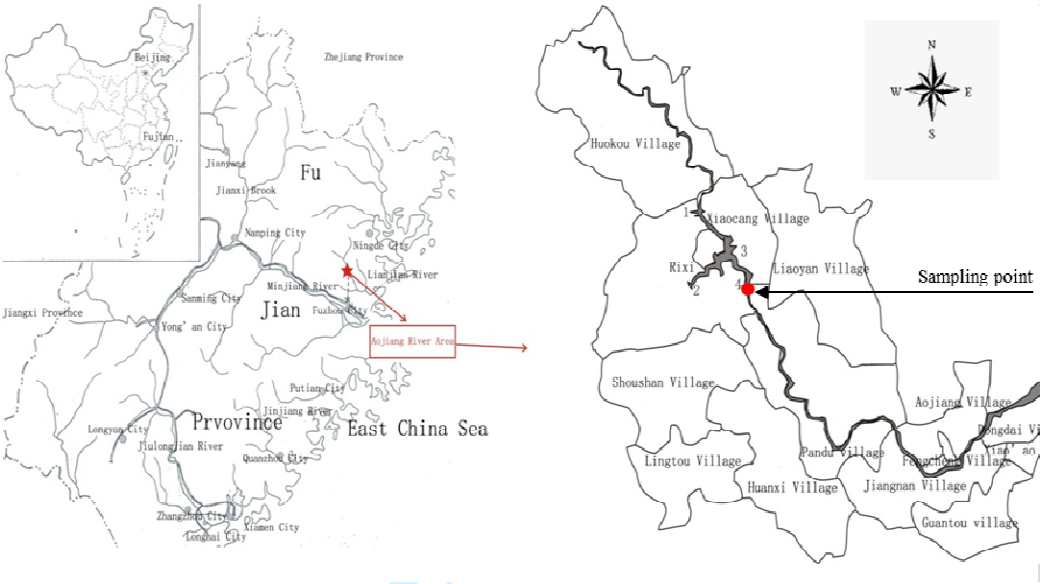
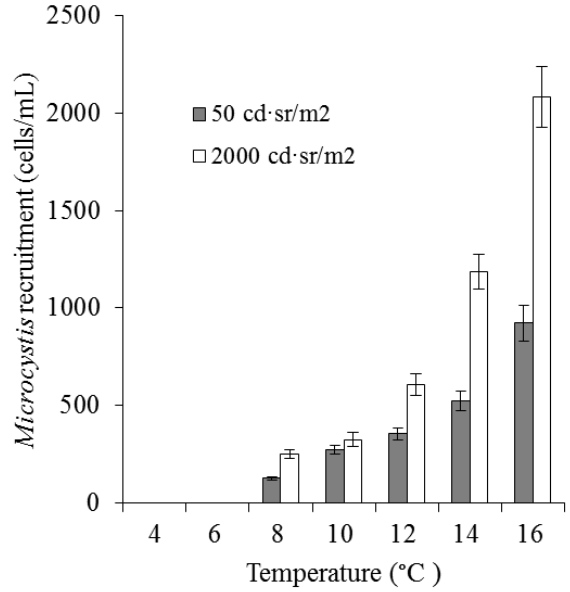
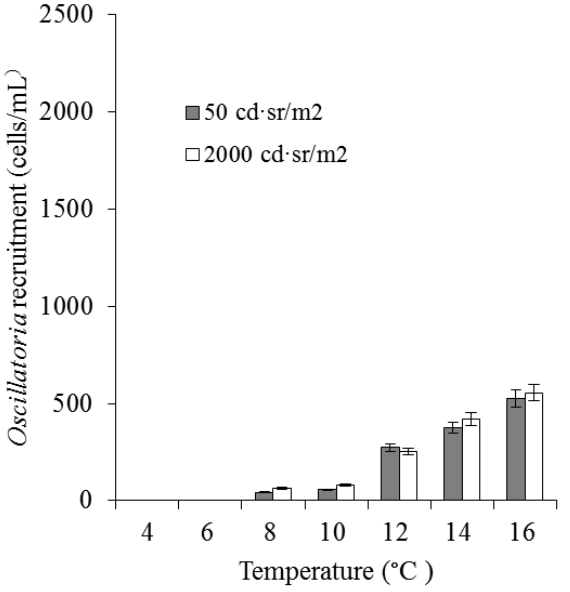


Figure 1. The location of Shanzi Reservoir and sampling point.

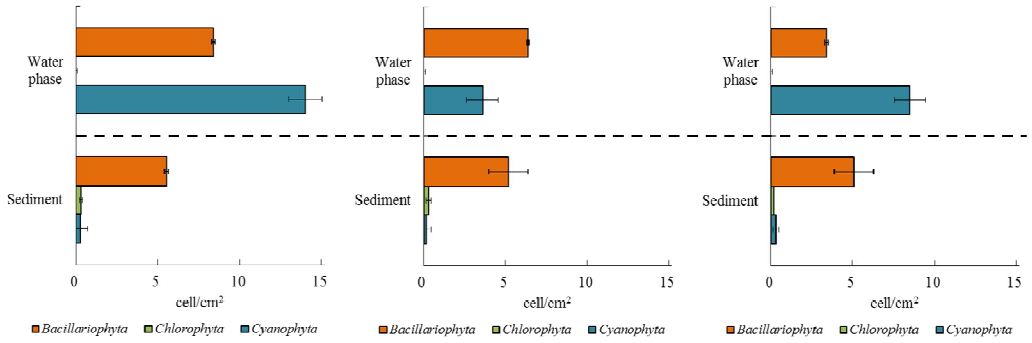


(a)



(b)

Figure 2. The recruitment of *Microcystis* (a) and *Oscillatoria* (b) from sediment samples of Shanzi Reservoir exposed to different temperature and light intensity. All the cyanobacteria were counted after 6 days cultivation in BG11 medium with a 12h:12h light-dark-cycle. The two treatments were of different light intensity during cultivation, 50 lx and 2,000 cd lx respectively.



(a) (b) (c)

Figure 3. Cyanobacterial community structure at water-sediment interface: original sediment (a), 50 lx treatment (b) and 2,000 lx treatment (c). The cyanobacteria numbers inside the sediment and water phase were analyzed by microscope after recruitment.

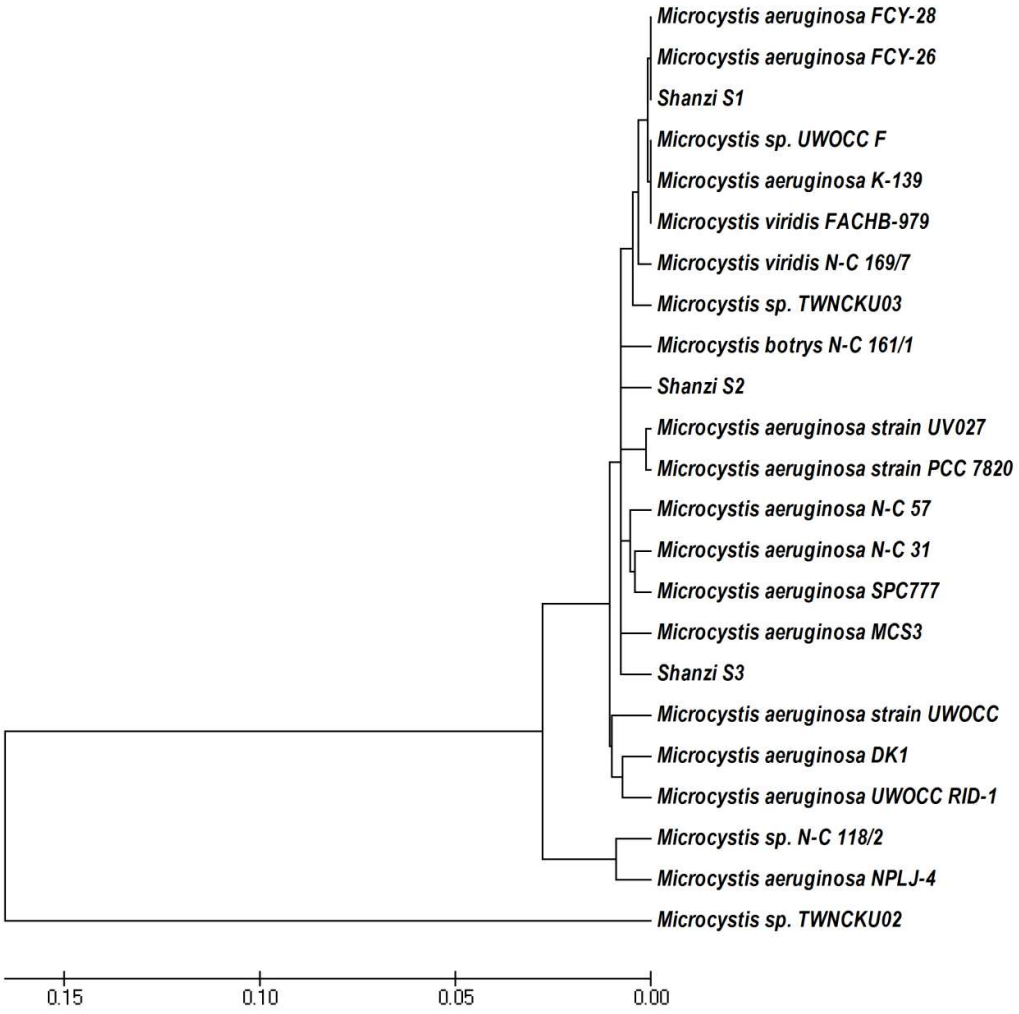


Figure 4. Phylogenetic analysis and Neighbour-Joining tree of *Microcystis* strains based on *mycA* gene.

Research on the recruitment of cyanobacteria from the sediment in the eutrophic Shanzi Reservoir

Yuping Su[1,2], Xuejing You[1], Houzhang Zhong[1], Hui Li[1], Yanfang Li[1], Ting Wang[1], Dayi Zhang[2]

1. Environmental Science and Engineering College, Fujian Normal University, Fuzhou, China PR, 350007.

2. Lancaster Environment Centre, Lancaster University, Lancaster, UK, LA1 4YW.

1. ANOVA analysis of *Microcystis* and *Oscillatoria* recruitment.

Table S1. ANOVA analysis of *Microcystis*.

	SS ($\times 10^4$)	dF	MS($\times 10^4$)	F	Fα	Significance level
Temperature	4152.4	1	4152.4	435.2	F0.05 (1,1)=161.40	*
Light (lx)	2175.9	1	2175.9	228.0	F0.01 (1,1)= 4052.40	*
Physical disturbance	832.5	1	832.5	87.3		
Nutrients	16.2	1	16.2	1.7		
Error e	9.5	1	9.5			

Table S2. ANOVA analysis of *Oscillatoria*.

	SS ($\times 10^4$)	dF	MS($\times 10^4$)	F	Fα	Significance level
Temperature	252.4	1	252.4	198.6	F0.05 (1,2) =18.51	**
Light (lx)	20.8	1	20.8	16.3	F0.01 (1,2) =98.50	
Physical disturbance	1.1	1	1.1			
Nutrients	12.2	1	12.2	9.6		
Error e	1.4	1	1.4			
eΔ	2.5	2	1.3			

SS=sum of squares of deviations; dF=degrees of freedom variance; MS=mean square; **=Significantly correlated; Round-off error eΔ.

2. Calibration curve of quantitative PCR

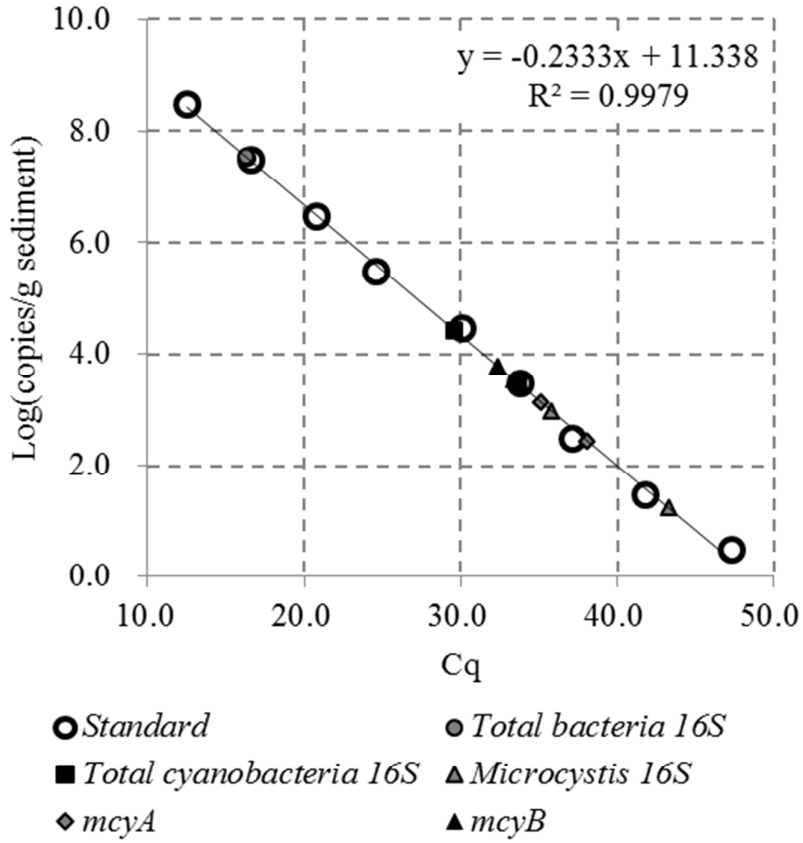


Figure S1. Calibration curve for quantitative PCR.